The two dimeric forms of RNase A

Salvatore Sorrentino[a], Roberto Barone[a], Enrico Bucci[b], Giovanni Gotte[c], Nello Russo[d], Massimo Libonati[c], Giuseppe D'Alessio[a,*]

[a] *Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II, Via Mezzocannone 16, 80134 Naples, Italy*
[b] *Centro di Studio di Biocristallografia del CNR, Via Mezzocannone 4, 80134 Naples, Italy*
[c] *Dipartimento di Scienze Neurologiche e della Visione, Sezione di Chimica Biologica, Università di Verona, Strada le Grazie 8, 37134 Verona, Italy*
[d] *Dipartimento di Scienze della Vita, Seconda Università di Napoli, Via Arena 18, 81100 Caserta, Italy*

Received 27 November 1999

Edited by Pierre Jolles

Abstract In 1965 Fruchter and Crestfield (J. Biol. Chem. 240, 2868–3874) observed that dimeric RNase A prepared by lyophilization from acetic acid could be separated into two forms. Surprisingly, no other structural or functional differences could be detected between the two forms. In 1998 a structure for dimeric RNase A was determined by X-ray crystallography by Liu et al. (Proc. Natl. Acad. Sci. USA 95, 3437–3442). We found that the two forms of dimeric RNase A have indeed different structural and functional properties, and suggest that the dimer whose structure was investigated by Liu and coworkers may be identified with the lesser form of dimeric RNase A.

Key words: Ribonuclease; RNase A; Protein oligomer

1. Introduction

In 1962 Crestfield et al. discovered that up to 20% of bovine pancreatic RNase A associated into dimers and higher aggregates upon lyophilization from concentrated solutions of acetic acid [1]. They then proposed an unusual structure for these dimers, based on an elegant, now historic experiment [2]. By associating two different types of inactive monomers, one inactivated by alkylation at His-12, located on the N-terminal helix, the other alkylated at His-119, located in the main protein body, enzymatic activity was restored; upon dissociation into monomers, activity was lost again. These results could only be explained by the formation upon dimerization of two composite active sites, each comprising His residues from different subunits. Their conclusion was that the dimeric structure was based on the interchange between protomers of their N-terminal helices.

This type of quaternary structure has been given increasing attention in the last few years, for its incidence in both artificial and natural oligomers [3], and for its evolutionary [4–6] and pathological [3] implications.

A few years after their initial observation on dimeric RNase A, Fruchter and Crestfield reported that upon ion-exchange chromatography on sulfo-ethyl Sephadex dimeric RNase A could be separated into two fractions [7], with the lesser one representing 20–25% of the dimeric protein. Surprisingly, after several tests, no structural or functional differences were detected between these two fractions.

Recently, the structure of dimeric RNase A was determined by X-ray crystallography [8]. It was found that indeed the two RNase A protomers associate in the dimeric structure through the exchange, or swap, of their N-terminal helices. However, it was not determined whether the investigated dimeric structure was that of the larger or of the lesser dimeric fraction.

The observation of the existence of two isoforms of dimeric RNase A has been recently confirmed and expanded, as isoforms were observed also for the higher aggregates of RNase A [9]. We report here that there are significant structural and functional differences between the two dimers of RNase A, and propose that the recently determined three-dimensional structure is that of the lesser dimeric isoform of the protein.

2. Materials and methods

2.1. Proteins

Bovine pancreatic RNase A (type XII-A) was purchased from Sigma. Bovine seminal RNase (BS-RNase) was purified as previously described [10]. Protein concentration was determined spectrophotometrically using for RNase A an absorbance coefficient of 7.3 at 280 nm for a 1% solution [11] and for BS-RNase an absorbance coefficient of 4.65 at 278 nm for a 1% solution.

2.2. Substrates

Yeast RNA, double-stranded poly(A)·poly(U), poly(U) and cytidylyl-(3′,5′)-adenosine (CpA) were Sigma products. The cytosolic RNase inhibitor (CRI) was purchased from Promega.

2.3. RNase assays

Ribonuclease activity toward yeast RNA was measured at 25°C following the Kunitz spectrophotometric assay [12] with 0.6 mg/ml of RNA in 0.1 M sodium acetate/acetic acid buffer, pH 5.0. Degradation of poly(U) and poly(A)·poly(U) at 25°C was followed spectrophotometrically at 260 nm in 0.1 M MOPS, pH 7.5 containing 0.1 M NaCl. Substrate concentration was 0.1 mM in phosphodiester groups (about 40 μg/ml). An enzyme unit was defined as the fraction of absorbance change per minute over the total measurable change [13]. Enzyme activity toward CpA was measured as described [14] in the absence or presence of RNase inhibitor with 50 μM substrate in 0.1 M MES at pH 6.0 containing 0.1 M NaCl and RNase free bovine serum albumin (10 μg/ml).

2.4. Cross-linking assay

The procedure described by Ciglic et al. [15] was followed. About 40 μg of each protein in 300 μl of 50 mM sodium acetate at pH 5.0

*Corresponding author. Fax: (39)-81-5521217.
E-mail: dalessio@unina.it

Abbreviations: RNase A, bovine pancreatic ribonuclease; D-I, the prevalent dimer-I fraction of dimeric RNase A; D-II, the dimer-II lesser fraction of dimeric RNase A; BS-RNase, bovine seminal RNase; DVS, divinyl sulfone; CRI, cytosolic RNase inhibitor

PII: S0014-5793(99)01742-1

containing 0.1% divinyl sulfone (DVS) was incubated at 30°C for 72 h. The reaction was stopped by addition of β-mercaptoethanol (200 mM). After 20 min, aliquots of 10 μl were analyzed with 15% SDS-PAGE under reducing conditions [16].

2.5. Preparation of RNase A dimers

Aggregates of ribonuclease A were obtained by lyophilization of the enzyme from 40% acetic acid solutions according to the procedure of Crestfield et al. [1]. The lyophilized material was dissolved in 1.2 ml of 0.2 M sodium phosphate buffer pH 6.55 and applied to a Sephadex G-75 column (1.5×105 cm) equilibrated with the same buffer and calibrated with monomeric RNase A and dimeric BS-RNase. Elution was performed at room temperature at a flow rate of 6 ml/h. The dimeric fraction was concentrated and dialyzed against buffer A (40 mM sodium phosphate buffer, pH 6.55) with Centriprep concentrators (Amicon), and applied to a Source 15 S HR 10/10 column equilibrated with buffer A on a Pharmacia FPLC system. The preparation of dimeric RNase A was resolved into two components by a phosphate gradient using 200 mM sodium phosphate, pH 6.55 as buffer B (see Fig. 1). Elution was performed at 25°C at a flow rate of 1 ml/min and fractions were manually collected.

2.6. Dissociation kinetics

Preparations of RNase A dimers (0.42 mg/ml), equilibrated in 50 mM Tris–HCl pH 7.5 containing 130 mM NaCl by extensive dialysis at 4°C, were incubated at 37°C. At appropriate time intervals, aliquots were analyzed by gel filtration with a flow rate of 0.6 ml/min on a Pharmacia FPLC system equipped with a Superdex 75 10/30 column equilibrated with 0.1 M Tris–HCl at pH 7.5 containing 0.3 M NaCl. The amounts of monomer and dimer were estimated by measuring the areas of their absorbance profiles at 278 nm.

2.7. Circular dichroism (CD) measurements

The CD measurements were made at 25°C on a 715 Jasco spectropolarimeter equipped with PTC-348 WI thermostat, under a constant nitrogen flow. Hellma quartz cells of 0.1 cm path length and a protein concentration of about 0.13 mg/ml were used in the far-UV region (190–240 nm). In the near-UV region (240–320 nm) protein concentration was 0.8 mg/ml with a path length of 1 cm. All samples were dissolved in 100 mM sodium phosphate buffer, pH 6.55. The reduced mean residue ellipticities ($[\theta]_{res}$) were calculated taking in account that the mean residue molecular weight for pancreatic RNase A is 110.5 [17].

3. Results and discussion

3.1. The two forms of dimeric RNase A

Dimeric RNase A was prepared by gel filtration and fractionated by chromatography on a cation exchanger as described in Section 2. Two main fractions were separated and collected (see Fig. 1). The main component, named dimer-I or D-I [7], appeared to have a higher positive net charge, as it eluted 7 min later than the minor one, named dimer-II or D-II. The latter represented 20–25% of the whole dimeric fraction of RNase A.

The homogeneity of each dimeric form was demonstrated by re-chromatography under the same conditions of suitable aliquots of purified D-I and D-II fractions. Each type of dimer eluted as a single peak with the same elution time as in Fig. 1. Upon dissociation by heating at 65°C for 30 min and re-chromatography, both D-I and D-II coeluted with monomeric RNase A. This confirms that D-I and D-II are distinct dimeric associations of a single monomeric form of RNase A.

The two small fractions preceding D-I and D-II, respectively, in the chromatographic pattern (see Fig. 1) were found to be dimeric by gel filtration (data not shown). After heating and dissociation, and upon re-chromatography as above, each eluted as a more negatively charged monomer,

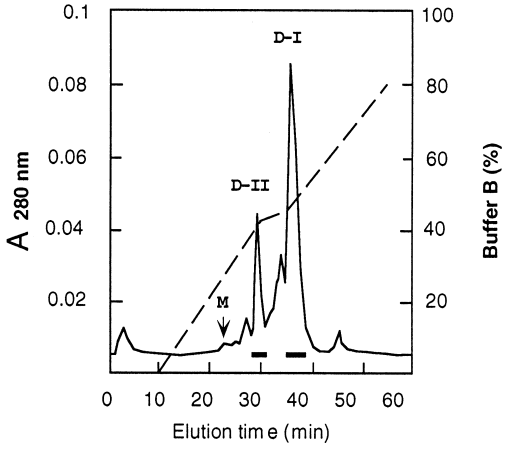

Fig. 1. Ion-exchange chromatography of RNase A dimers. An aliquot (about 2 mg) of RNase A dimers from Sephadex G-75 was applied to a Source 15 S HR 10/10 column (Pharmacia FPLC system) equilibrated with 40 mM sodium phosphate buffer, pH 6.55 (buffer A). Elution was performed with a gradient as indicated (dashed line), using 200 mM sodium phosphate, pH 6.55 as buffer B, at a flow rate of 1 ml/min at 25°C. The elution time (23 min) of native RNase A (M) is indicated by an arrow.

1 min earlier than native RNase A. This suggests that they are the respective deamidation products of the two main dimers D-I and D-II. Possibly, as found for naturally dimeric BS-RNase when compared to its monomeric counterpart [18], deamidation is faster in dimeric than in monomeric RNase A.

Based on these results, we can conclude that the two dimeric forms D-I and D-II are those originally described by Fruchter and Crestfield [7] and recently investigated by Gotte et al. [9].

3.2. Catalytic and structural properties of D-I and D-II of RNase A

The catalytic activities of D-I and D-II were compared in side-by-side assays performed with single- and double-stranded polyribonucleotides. Native monomeric RNase A was included as a standard for comparison. The results are summarized in Table 1. On yeast RNA and on poly(U) as substrates D-I and D-II displayed similar activity values, somewhat lower than that measured for RNase A. On the double-stranded substrate poly(A)·poly(U) instead the two dimers were both more active than the monomer, with D-I twice as active as D-II.

Table 1
Activity of RNase A and its dimeric forms on single- and double-stranded polyribonucleotides

	Specific activity (U/mg of protein)[a] toward		
	Yeast RNA[b]	Poly(U)[c]	Poly(A)·Poly(U)[c]
RNase A	112 ± 6	395 ± 20	1.3 ± 0.1
D-I	88 ± 4	382 ± 13	6.2 ± 0.3
D-II	85 ± 4	387 ± 15	2.5 ± 0.1

[a]Spectrophotometric assays performed at 25°C. Each activity value is the mean (± S.D.) of four measurements. Units are defined in Section 2.
[b]Kunitz assays performed at pH 5.0 in sodium acetate buffer.
[c]Assays performed at pH 7.5 in 0.1 M MOPS containing 0.1 M NaCl.

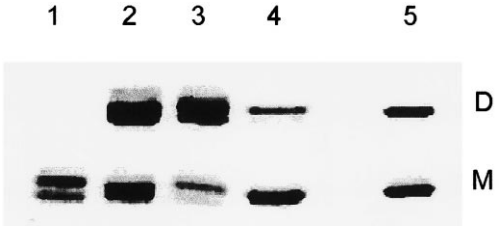

Fig. 2. SDS-PAGE showing the cross-linking of subunits of dimeric RNases using DVS, with RNase A as a negative control. The proteins were reacted with DVS for 72 h at 30°C. Lane 1, native RNase A; lane 2, RNase A D-I; lane 3, RNase A D-II; lane 4, BS-RNase. Native RNase A (M) and native BS-RNase (D) were added as standards (lane 5). The gel was stained with Coomassie blue.

These results are in line with those recently obtained with different assay procedures [9]. Taken together with the observations reported above that both D-I and D-II have a positive net charge higher than that of monomeric RNase A, and that D-I is more basic than D-II, they support the proposal [13] that the action of pancreatic-type RNases on double-stranded substrates correlates with positive charge densities located in discrete regions of the protein.

The structures of the CRI [19] and of its complexes with RNase A [20] or angiogenin [21] have been determined. When the structure of dimeric RNase A [8] was modeled onto that of CRI (data not shown) it was apparent that dimeric RNase A cannot bind to the inhibitor. As the structure determined for dimeric RNase A [8] must be clearly assigned to at least one of the two dimers D-I or D-II, their sensitivity to CRI was tested by assaying their enzymatic activity in the presence of CRI.

With CpA as a substrate both D-I and D-II dimers were found to be fully susceptible to inhibition by CRI. Based on the considerations expressed above, that at least one of the dimeric forms cannot bind to the inhibitor, and given the metastability of the two dimers (see below), these results suggest that both dimeric isoforms dissociate upon interacting with CRI, and bind to the inhibitor as monomers. Yet, it cannot be excluded that the dimeric form distinct from that described by Liu et al. [8] can also fit as a dimer in the complex with CRI.

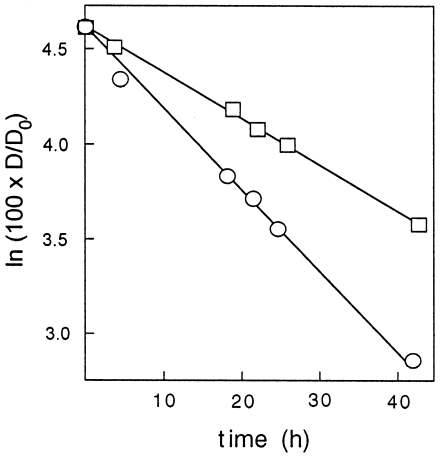

Fig. 3. Time course of dissociation of D-I and D-II. The two dimers were incubated at 37°C in 50 mM Tris–HCl, pH 7.5, containing 130 mM NaCl. At various times, the two samples were analyzed by gel filtration to reveal monomer formation. D_0 and D indicate dimer concentrations at t_0 and at the indicated time, respectively. Circles and squares refer to D-I and D-II, respectively.

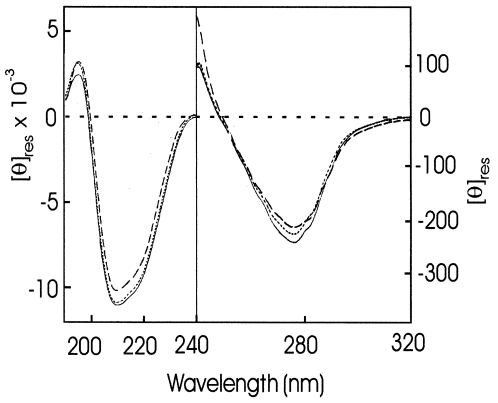

Fig. 4. Far-UV (190–240 nm) and near-UV (240–320 nm) CD spectra of RNase A (solid line) and its dimeric forms, D-I (dashed line) and D-II (dotted line).

It has been shown that cross-linking with DVS is a convenient tool for determining whether RNase dimers exchange their N-terminal arms as they do in seminal RNase [15]. The assay is based on the selective simultaneous reactivity with DVS of both His-12, located on the N-terminal arm of pancreatic-type RNases, and His-119, located on the main protein body. When in an RNase dimer there is exchange of the N-terminal arms between protomers, the two His residues are contributed one from each protomer, and the result of the reaction with DVS is a covalent link between protomers. In the absence of arm exchange, just as in a monomer, His-12 and -119 belonging to the same protomer are instead cross-linked.

D-I and D-II were treated for 72 h at 30°C with DVS as described [15]. Cross-linking was tested by SDS-PAGE under reducing conditions. Positive (BS-RNase), and negative (RNase A) controls were included in the experiment. As shown in Fig. 2, about 60% of D-I was found to migrate as a cross-linked dimer, whereas D-II was almost completely cross-linked. These results lead to the conclusion that in both D-I and D-II the protomers exchange their N-terminal helices, as found for the dimer investigated by Liu et al. [8]. The observed fragmentations, with band splitting (see Fig. 2), are side effects of the reaction [15].

Given the metastable nature of RNase A dimers (see below), it is not surprising that both D-I and D-II in part dissociate and react with DVS as monomers, as has already been reported for the unresolved mixture of dimers [15]. Hence this difference in reactivity with DVS between D-I and D-II can be explained by a different degree of dissociation of the two dimers in the prolonged incubation with DVS at 30°C.

This interpretation is confirmed by the results of an experiment in which the kinetics of dissociation of the two dimers at 37°C were measured. The fraction of each dimer dissociated into monomers as a function of time was determined by gel filtration (see Section 2). D-I was found to be more readily dissociated than D-II (see Fig. 3). The data fitted first-order plots and gave $t_{1/2}$ times of 29 and 16 h, respectively, for D-II and D-I. The different stabilities of D-I and D-II indicate that the two dimers have a different structure.

Table 2
Secondary structure content of D-I and D-II, as evaluated from CD measurements, and comparison with the secondary structure content determined for monomeric and dimeric RNase A by X-ray crystallographic analyses

	Assignments (%) from crystallographic data		Assignments (%) from CD data		
	RNase A [24]	Dimeric RNase A [8]	RNase A	D-I	D-II
α-Helix	17.7	20.5	17.0	9.5	16.4
β-Structure	33.0	33.0	34.7	38.4	34.2
β-Turns	16.9	14.5	16.8	17.5	17.6
Random coil	32.4	32.0	31.5	34.6	31.8
Total	100.0	100.0	100.0	100.0	100.0

Previous data in the literature have led to the conclusion that the two dimers cannot be distinguished in their thermostability [7]. In these assays, however, the stability of the dimers was measured at high temperatures (40–65°C), and in a phosphate buffer, which in the same report was found to stabilize the associated dimers [7]. Thus the different assay protocol explains the discrepancy between the results of the two assays.

Finally, the two dimers were analyzed by CD. Fig. 4 shows the far-UV and the near-UV spectra of D-I and D-II, compared to the spectra recorded for monomeric RNase A under identical conditions. The differences in the CD profiles between the spectra of the two dimers and their differences with those of RNase A are not large, but clearly significant and reproducible after repeated analyses. The spectra of D-II appear in fact to be almost superimposable to those of monomeric RNase A, but for bands of higher intensity at 195 and at about 278 nm. Those of D-I instead show a loss of secondary structure in the region around 210 nm, and a much higher positive band at 240 nm. The latter feature is suggestive of a major exposure in D-I of the phenolic rings of tyrosine residue(s) [22].

A quantitative analysis of the CD data obtained for the two dimers in terms of secondary structure content was performed with the deconvolution procedure of Böhm et al. [23], using a set of 34 proteins including RNase A. The results, summarized in Table 2, indicate that the percentage values calculated for the α-helix, the β-structure, and the random coil conformations from the spectra of D-II are very close to the corresponding values calculated with the same procedure for RNase A. In D-I instead a much lower content in α-helix is found, compared with both D-II and native monomeric RNase A.

These values were also compared (see Table 2) with the corresponding percent values of α-helix, β-structure, turns and coil conformations calculated from the structures determined by the X-ray crystallographic analyses of dimeric [8] and monomeric [24] RNase A. These values were obtained with the PROCHECK procedure, as implemented with the Kabsch and Sander algorithm [25], including for the helix and β-structure contents only residues with correct ϕ-ψ angles and hydrogen bonding. This comparison shows very clearly that the secondary structure of D-I is completely different from that of the dimer investigated by Liu et al. [8], especially for its much lower α-helix content (9.5 versus 20.5%) and its higher content of β-structure (38.4 versus 33.0%). The values for D-II instead, as calculated with the deconvolution procedure, are much closer or identical to those of the RNase dimeric form whose structure is known. It may thus be proposed that the dimeric form of RNase A for which the three-dimensional structure is available [8] is that of the lesser dimer (D-II).

3.3. Conclusions

The results reported here lead to the following conclusions. Upon dimerization, RNase A can associate into two different dimeric forms, as originally observed by Fruchter and Crestfield [7], and recently confirmed [9]. These two forms have different activities on double-stranded RNA substrates, different thermostabilities, and different, albeit similar, structures. The analysis of CD data strongly suggests that the lesser dimer (D-II), with a higher content of swap of N-terminal segments between subunits and a higher thermostability, is the dimeric form for which the three-dimensional structure has been determined [8]. Recently, a comparison of X-ray crystallographic data has suggested that the structure investigated by Liu and co-workers [8] is indeed that of the lesser isoform of dimeric RNase A (Y. Liu, personal communication).

Acknowledgements: The authors wish to acknowledge grants from the Ministero dell'Università e della Ricerca (PRIN-SMIP 1997) and from MURST-CNR (programma L. 95/95).

References

[1] Crestfield, A.M., Stein, W.H. and Moore, S. (1962) Arch. Biochem. Biophys. 1 (Suppl.), 217–222.
[2] Crestfield, A.M., Stein, W.H. and Moore, S. (1963) J. Biol. Chem. 238, 2421–2428.
[3] Schlunegger, M.P., Bennet, M.J. and Eisenberg, D. (1997) Adv. Protein Chem. 50, 61–122.
[4] Bennett, M.J., Schlunegger, M.P. and Eisenberg, D. (1995) Protein Sci. 4, 2455–2468.
[5] D'Alessio, G. (1999) Progr. Biophys. Mol. Biol. 72, 271–298.
[6] D'Alessio, G. (1999) Eur. J. Biochem. 266, 1–11.
[7] Fruchter, R.G. and Crestfield, A.M. (1965) J. Biol. Chem. 240, 3868–3874.
[8] Liu, Y., Hart, P.J., Schlunegger, M.P. and Eisenberg, D. (1998) Proc. Natl. Acad. Sci. USA 95, 3437–3442.
[9] Gotte, G., Bertoldi, M. and Libonati, M. (1999) Eur. J. Biochem. 265, 680–687.
[10] Tamburrini, M., Piccoli, R., De Prisco, R., Di Donato, A. and D'Alessio, G. (1986) Ital. J. Biochem. 35, 22–32.
[11] Wang, D. and Moore, S. (1977) Biochemistry 16, 2937–2942.
[12] Kunitz, M. (1946) J. Biol. Chem. 164, 563–568.
[13] Sorrentino, S. and Libonati, M. (1994) Arch. Biochem. Biophys. 312, 340–348.
[14] Witzel, H. and Barnard, E.A. (1962) Biochem. Biophys. Res. Commun. 7, 295–299.
[15] Ciglic, M.I., Jackson, P.J., Raillard, S.A., Haugg, M., Jermann, T.M., Opitz, J.G., Trabesinger-Ruf, N. and Benner, S.A. (1998) Biochemistry 37, 4008–4022.
[16] Laemmli, U.K. (1970) Nature 227, 680–685.
[17] Puett, D. (1972) Biochemistry 11, 1980–1990.

[18] Di Donato, A., Ciardiello, M.A., de Nigris, M., Piccoli, R., Mazzarella, L. and D'Alessio, G. (1993) J. Biol. Chem. 268, 4745–4751.
[19] Kobe, B. and Deisenhofer, J. (1993) Nature 366, 751–756.
[20] Kobe, B. and Deisenhofer, J. (1996) J. Mol. Biol. 264, 1028–1043.
[21] Acharya, K.R., Papageorgiou, A.C. and Shapiro, R. (1997) EMBO J. 16, 5162–5177.
[22] Grandi, C., D'Alessio, G. and Fontana, A. (1979) Biochemistry 18, 3413–3420.
[23] Böhm, G., Muhr, R. and Jaenicke, R. (1992) Protein Eng. 5, 191–195.
[24] Wlodawer, A., Svensson, L.A., Sjolin, L. and Gilliland, G.L. (1988) Biochemistry 27, 2705–2717.
[25] Kabsh, W. and Sander, C. (1983) Biopolymers 22, 2577.